DETAILED PROJECTION PATTERNS OF SEPTAL AND DIAGONAL BAND EFFERENTS TO THE HIPPOCAMPUS IN THE RAT WITH EMPHASIS ON INNERVATION OF CA1 AND DENTATE GYRUS

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Detailed Projection Patterns of Septal and Diagonal Band Efferents to the Hippocampus in the Rat With Emphasis on Innervation of CA1 and Dentate Gyrus

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NYAKAS, C., P. G. M. LUITEN, D. G. SPENCER AND J. TRABER. *Detailed projection patterns of septal and diagonal band efferents to the hippocampus in the rat with emphasis on innervation of CA1 and dentate gyrus.* BRAIN RES BULL **18**(4) 533–545, 1987.—The detailed patterns of afferentation to the ammon's horn and dentate gyrus of the hippocampus in the rat were investigated employing the anterograde tracer *Phaseolus vulgaris* leuco-agglutinin (PHA-L) after punctate iontophoretic injections in the medial septum (MS) and vertical limb of the diagonal band of Broca (VDB). The topographically ordered innervation pattern was different in the regio superior (or CA1) vs. the regio inferior (or CA3) and in the dorsal vs. ventral aspects of ammon's horn and dentate gyrus. The CA1 pyramidal and dentate granule cell layers in the dorsal hippocampus received afferent input almost exclusively from the VDB, whereas those cell layers in ventral hippocampus were supplied from both VDB and MS. The PHA-L labeled projecting fibers could be differentiated into two distinct fiber systems. One class of thick and coarse axons (tentatively called type I fibers) carried fewer but larger terminal boutons and were found to infiltrate the entire stratum oriens, dentate hilus, all layers of the regio inferior and the CA1 str. moleculare. A second, delicate thin (type II) fiber system provided with numerous and passant varicosities showed a much more restricted laminar innervation pattern and appeared to originate from areas in MS-VDB which are rich in AChE-positive neurons. The densest type II fiber networks could be observed in the CA1 subpyramidal and dentate supragranular zones, in the CA1 stratum lacunosum-moleculare and in the dentate middle third molecular layer. This laminar type II innervation pattern showed a remarkable coincidence with the reported distribution of cholinergic marker enzymes. The topographic and spatial organization of the projections described above will be discussed in relation to their possible functional significance.

PHA-L anterograde tracing Medial septum Vertical diagonal band of Broca Projection patterns CA1 Dentate gyrus AChE-positive neurons

OF the massive extrinsic sources of input to the hippocampus, i.e., entorhinal cortex and septum, the latter originates mainly in the nuclei of medial septum (MS) and the vertical diagonal band of Broca (VDB) [18, 21, 22, 24, 40, 45]. The partly cholinergic nature of septal afferentation has been well established [1, 18, 22, 26, 43, 50] and gained support from early studies with lesions in the medial septum [25,42] or transection of septo-hippocampal connections [18]. Massive electrolytic lesions of the septum practically abolish hippocampal acetylcholine synthetizing and degrading enzyme activities and acetylcholine content (see for review [43]). These studies posed more detailed questions on the exact origin of extrinsic and the limited intrinsic cholinergic innervation of the hippocampus. More recently, the extensive research on the forebrain cholinergic system in relation to the pathogenesis of dementia of Alzheimer type renewed interest in understanding more precisely the origin and termination patterns of extrinsic neurons projecting to telencephalic structures such as cerebral cortex and hippocampus.

Research based on techniques using a combination of retrogradely transported intra-axonal markers and histochemical demonstration of acetylcholine esterase (AChE) [22,26] or immunocytochemical localization of choline acetyltransferase (ChAT) [1, 18, 38, 50] positive neurons has more precisely defined the origin of cholinergic neurons in MS-VDB. The same studies also revealed that a considerable

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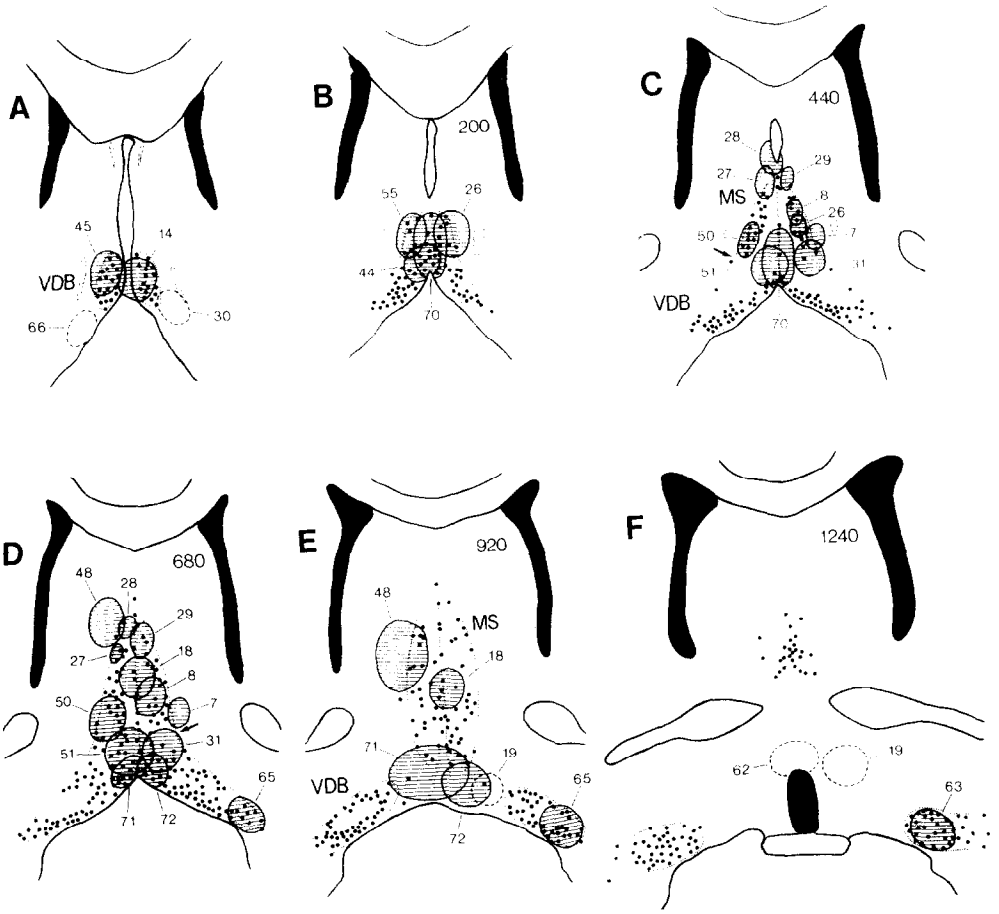


FIG. 1. Extension and location of injection sites (hatched areas) centered in the nuclei of medial septum (MS) and vertical diagonal band (VDB). Control cases outside of the MS-VDB system with no hippocampal projection are encircled with broken lines. The series of coronal sections (A to F) are camera lucida drawings from a DFP-treated animal with the distribution of AChE positive neurons indicated with the black dots. Distances in microns from the first section (A) are indicated in the right upper corner of each section.

proportion of MS-VDB neurons projecting to the hippocampus are non-cholinergic in nature [1,50].

Our current knowledge of the laminar arrangement of terminal fields of cholinergic septal afferentation to the hippocampus is still largely influenced by the early analyses of AChE stained brain sections [9] or neurochemical measurement of AChE and ChAT [11]. Recently, with immunocytochemical techniques employing monoclonal antibodies to ChAT, a ChAT-positive lamination became visible in the dentate gyrus and hippocampus proper [12,15], which closely corresponds to the previously described distribution of AChE. The link between the origin and terminal arborization of septal afferentation including the cholinergic neurons, however, is still poorly understood despite results obtained with techniques using anterograde degeneration [25, 29, 36] or anterograde transport of labeled amino acids [21, 27, 37]. This is mainly due to their inherent methodological limitations such as fibers-of-passage problems in degeneration procedures and the lack of distinction between fibers and terminals in ^{3}H-amino acid tracing. Furthermore, the diffusion of radioactive amino acids at the injection site obscures the precise localization of effective tracer uptake area.

The more recent anterograde tracing technique applying *Phaseolus vulgaris* leuco-agglutinin (PHA-L) as a transported intra-axonal marker has the advantage of tracing neurons, their axons and the entire synaptic terminal fields at light microscopic levels [13, 20, 46]. The aim of the present study was to label a limited number of neurons in a well defined area of MS-VDB complex and study the terminal field of the projections in the entire hippocampus specifically in relation to the laminar arrangement of cholinergic markers as has been reported in detail in the recent literature [12, 15, 44]. The injection areas were mapped in parallel sections stained for AChE positive cellbodies in order to relate the tracer injection site to distribution of AChE-rich somata within the boundary of the injection site. In the present study we restricted ourselves to describe the projections to the hippocampus proper and dentate gyrus, although neurons located in the injected areas also sent considerable amounts of fibers and terminals to other cortical areas including the subiculum, entorhinal and cingulate cortex, the olfactory bulb, as well as to a variety of subcortical structures and will be dealt with in a separate report.

Since the septo-hippocampal pathway is topographically organized along the septo-temporal axis of the hippocampus

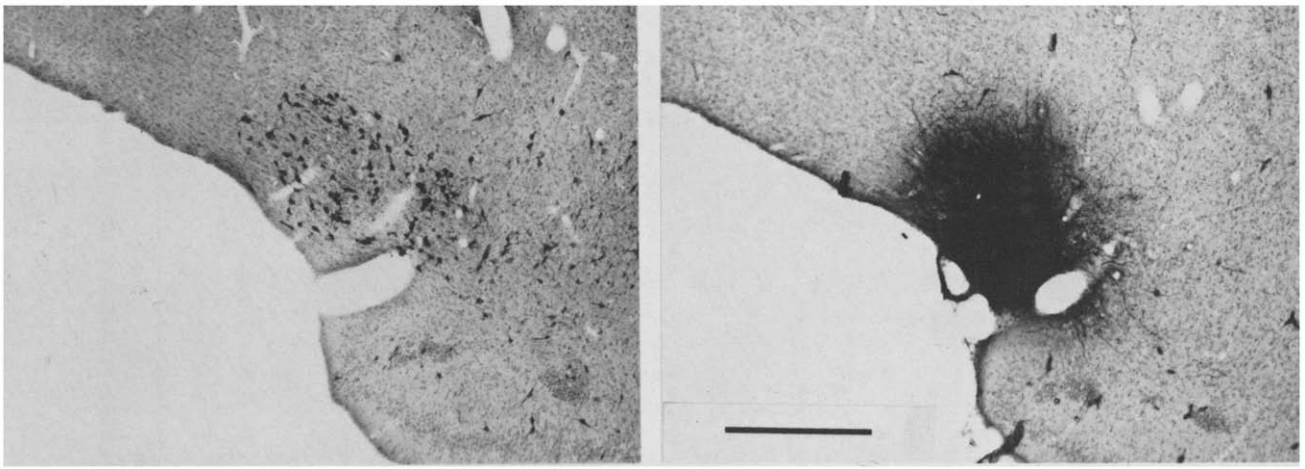


FIG. 2. The left panel photomicrograph shows the distribution of AChE-rich cell bodies in the posterior aspects of the nucleus of the vertical limb of the diagonal band of Broca. The right panel is a micrograph from the adjacent section with a *Phaseolus vulgaris* leuco-agglutinin injection in the VDB area (compare to Exp. 63 in Fig. 1). Scale bar=500 μm.

[24, 27, 28], we discriminated in terminal projection patterns in the dorsal versus ventral hippocampus. Regarding the source of PHA-L immunostained fibers, a further distinction could be made between the two main subdivisions of the hippocampus proper. Special attention was paid to the innervation patterns in the regio superior, partly because the organization of septal afferents to this sector of the ammon's horn is a matter of considerable dispute in the literature [14, 33, 37], and partly because the innervation patterns follow the laminar arrangement more clearly here than in the regio inferior.

Beside CA1, the laminated innervation patterns will also be surveyed in CA3 and dentate gyrus. Both CA1 and dentate gyrus house the generators of the theta rhythm (rhythmic slow electrical activity), and considerable electrophysiological research efforts are aimed at localizing the theta generators into the known laminated anatomical structures of these regions [5, 14, 28, 31, 52].

In tracing the efferent connectivity from MS-VDB to the hippocampus, a distinction could be made between two morphologically different fiber systems which may have important functional implications. A description will be presented of a very thin and delicate fiber network which gives numerous synaptic boutons around the somata and dendritic fields of the two main neuron types, i.e., pyramidal and granule cells. The source of this probably cholinergic afferentation appears to follow topographic arrangements in the MS-VDB complex.

METHOD

Animals

The experiments were carried out on 37 male Wistar rats between 3 and 4 months of age. Thirty six animals received one *Phaseolus vulgaris* leuco-agglutinin (PHA-L) injection each into the MS or VDB region. Before sacrifice, the animals received an injection of diisopropylfluorophosphate (DFP). Every third brain section was processed for histochemical demonstration of AChE-positive neurons in order to determine their overlap with the PHA-L injection spots (Figs. 1, 2). Adjacent sections were treated for immunocytochemical visualization of PHA-L. One animal was injected with DFP only and processed for AChE activity to gain a complete survey on putative cholinergic neurons of the MS-VDB complex.

PHA-L Procedure

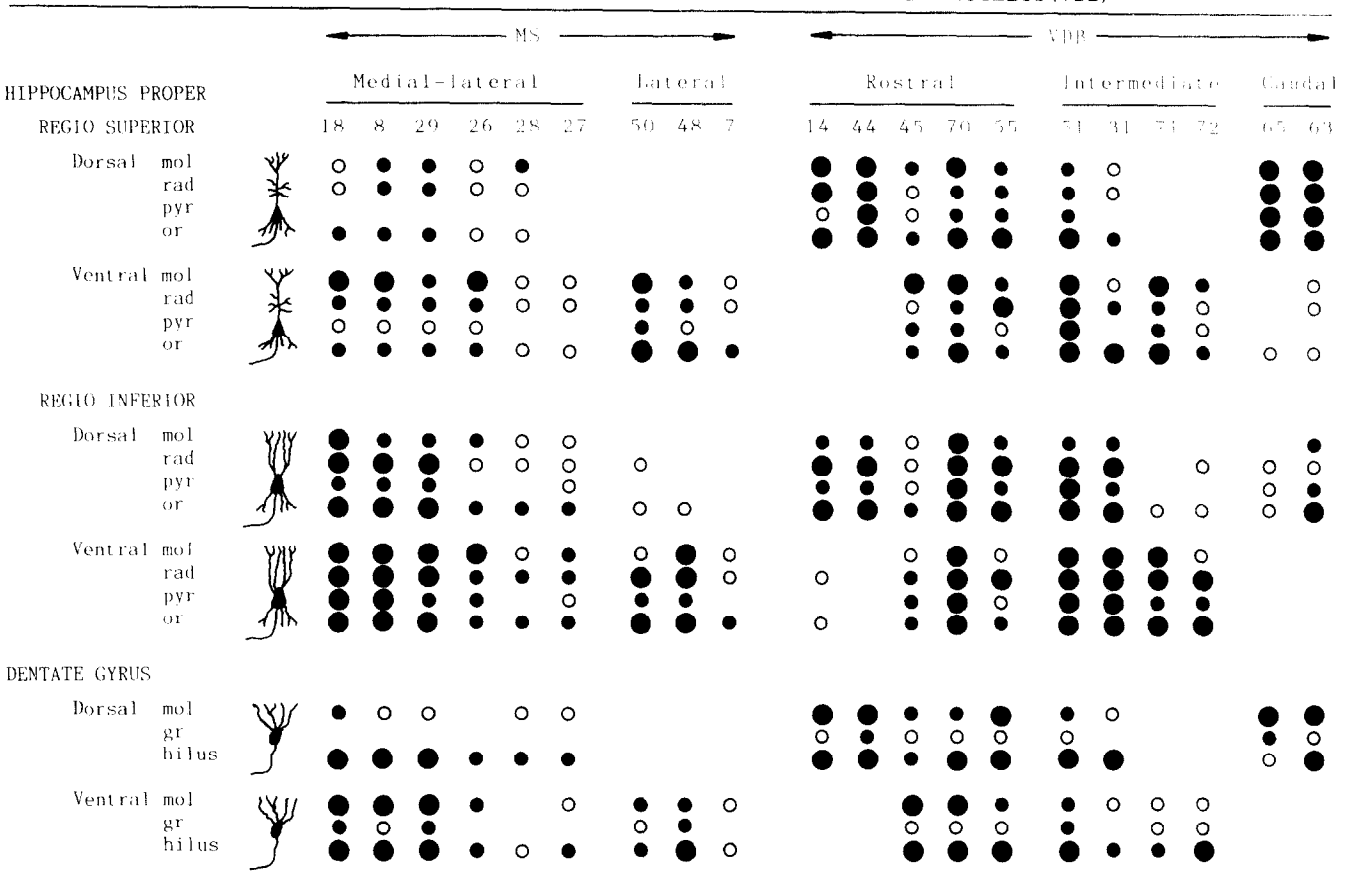
PHA-L injection and immunostaining procedures have been described in greater detail in previous reports [20,46]. Briefly, under combined anesthesia of Hypnorm (Duphar, 0.4 mg/kg IM) and pentobarbital (sodium salt, 30 mg/kg IP) PHA-L deposits were delivered via stereotaxically positioned micropipettes [35]. These bevelled glass micropipettes with tip diameters of 18–23 μm were filled with a solution of 2.5% PHA-L (Vector Labs.) in TRIS-buffered saline (TBS, pH=7.4) and connected to the positive pole of a constant current source (Midgard CS 3). The current intensity for iontophoretic delivery of the tracer varied between 5.0–6.0 μA and was applied from 45 min in a 7 sec on, 7 sec off cycle. After a survival time of 7–9 days the brain tissue was fixed by transcardial perfusion with a mixture of 2.5% glutaraldehyde, 0.5% paraformaldehyde and 4% sucrose in 0.05 M phosphate buffer (pH=7.4). After dehydration in 30% sucrose and sectioning at 40 μm on a cryostat microtome, the sections were thoroughly rinsed in TBS and processed for immunocytochemical staining of PHA-L distribution [13,46]. Incubations were carried out at room temperature in the dark in an incubation solution consisting of 0.5 M NaCl, 0.5% Triton X-100 and 0.05 M TRIS buffer (pH=8.6). Incubation with goat-anti-PHA-L (Vector Labs.) 1:2000 was done for 48 hrs, rabbit-anti-goat IgG (Sigma) 1:200 for 20–24 hr and goat peroxidase-antiperoxidase complex (DAKO) 1:400 for 4 hr. The sections were stained for peroxidase in 40 mg diaminobenzidine (Sigma) in 100 ml TRIS buffer at pH=7.6 and 0.9 ml 1.5% H_2O_2 for 40–60 min. The sections were mounted and counterstained with cresylviolet. The PHA-L processing was carried out on every third section and combined PHA-L/AChE or AChE staining on every sixth adjacent section.

AChE Procedure

For mapping of AChE-positive neurons in the MS-VDB complex a pharmaco-histochemical technique was used including DFP pretreatment according to the method described by Butcher *et al.* [9]. DFP (Sigma) dissolved in

TABLE 1

SEMIQUANTITATIVE EVALUATION OF TERMINAL PROJECTIONS TO THE HIPPOCAMPUS FROM INJECTION SITES LOCATED IN THE MEDIAL SEPTAL NUCLEUS (MS) AND THE VERTICAL DIAGONAL BAND NUCLEUS (VDB)



Symbols: ● massive, • moderate, ○ sparse,—no innervation. The molecular layer in the hippocampus proper includes the stratum lacunosum. Hilus comprises CA4 and the polymorph layer.

arachis oil (1:1000) was injected IM in a dose of 1.5 mg/kg body weight, which was immediately followed by an IP injection of 5 mg/kg atropine. After 5 hr survival time the above described PHA-L procedure started with perfusion and fixation of the brain. For AChE staining the technique of Karnovsky and Roots [17] was followed as described elsewhere. In the combined PHA-L and AChE staining the sections were first stained for AChE prior to processing for PHA-L.

Of the 36 PHA-L experiments 7 cases were discarded because of technical failures. Another 9 injections were centered beyond the boundary of the MS-VDB complex and thus negative for hippocampus labeling. The remaining 20 experiments were evaluated and analyzed for anterogradely labeled terminal efferents to the hippocampus and are summarized in Fig. 1 and Table 1. It should be mentioned that cortical afferents other than to the hippocampus, as well as the descending brainstem projections will not be further dealt with in the present paper.

RESULTS

Nomenclature

Medial septum and vertical diagonal band of Broca. The extent of the MS-VDB complex as a whole can easily be delineated, but the transition of the two composing nuclei remains obscure. In agreement with earlier reports [26,45], the boundary between MS and VDB was ventral to the lateral mass of MS cholinergic neurons where the AChE-rich cell layer becomes sparse (see arrow in Fig. 1C). More posteriorly, the boundary between MS and VDB was defined just dorsal to where the VDB appears as a ring-like configuration (Fig. 1D, 1E). The location and extension of PHA-L injections and subsequent labeled projection patterns allow us to make some additional distinctions, to be discussed below. The VDB is a more elongated structure in the anterior-to-posterior plane than the MS, which continues caudally close to the ventromedial brain surface (ventral VDB, see Fig. 1E and 1F). The VDB approaches the horizontal limb of the diagonal band approximately at the level of the crossing of the anterior commissure [2,53].

As has been clearly demonstrated previously [26] the cholinergic neurons within the MS of the rat form a midline raphe consisting of a small number of neurons, and a substantial mass of such cells situated in the lateral half of the nucleus. Between these two cell populations there is a contingent of neurons that are apparently non-cholinergic.

Hippocampus. The nomenclature applied to the various subdivisions, layers and cytoarchitectonic features of the hippocampus follows previous descriptions [2, 10, 19, 33,

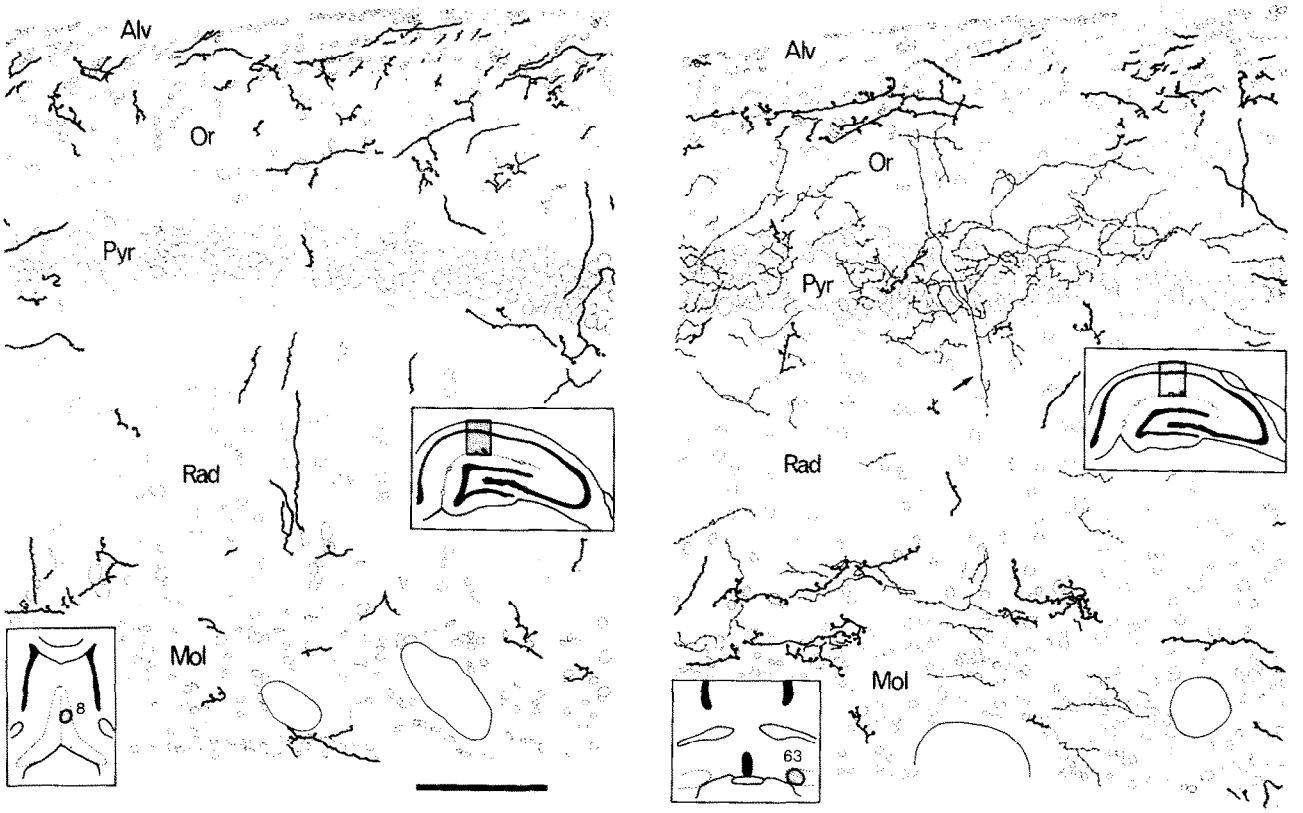


FIG. 3. Afferentation to the regio superior (CA1) of the dorsal hippocampus from the MS (left, Exp. 8) and VDB (right Exp. 63). Note the absence (8) and the presence (63) of a fine fiber network with numerous synapses around and inside the pyramidal layer. Arrow (right) points to a passing-through fiber which seems to be heading towards a neighbouring network in the molecular layer. Coarse fibers with heavy boutons supply mainly stratum oriens and the deep part of the molecular layer. The main targets of these axons appear to be interneurons. Calibration bar=100 μm.

54]. The hippocampus proper (cornu ammonis) is divided into two regions, the regio superior and inferior as defined by Cajal [10]. The nomenclature of Lorente de No [19] as CA1 for regio superior is also used here. The regio inferior comprises CA3 and the transition area CA2. The CA4 region is included in the hilus of the fascia dentata for the sake of simplicity.

Concerning the layers of hippocampus proper a stratum moleculare, radiatum, pyramidale, and oriens can be distinguished. The stratum lacunosum is included into the stratum moleculare since it is indistinguishable in Nissl stained material. In the dentate gyrus, the MS-VDB efferents could be demonstrated in three layers: stratum moleculare, granulare, and the hilus including the polymorph layer and CA4. To indicate regions along the longitudinal axis the terminology of dorsal (septal) and ventral (temporal) is used. To describe directions we adapted the terminology from Zimmer and Haug [54]: superficial means towards the hippocampal fissure and deep means towards the alveus or hilus.

Location of Injection Sites

The size and location of the PHA-L injections described in the present study are shown in Fig. 1 (A–F). The combined AChE and PHA-L staining techniques allowed us to relate the extension of injection sites to the location of neuronal cholinergic markers, although it should be noted that AChE is also found in some non-cholinergic cells of the MS-VDB complex. Those injections which showed overlap with AChE-rich cell bodies did result in anterograde labeling to the hippocampus. However, injections which did not coincide with the MS-VDB system in all cases were negative for hippocampal projections (injection sites delineated with broken lines). From these so-called negative cases, 5 are indicated in Fig. 1 for reference. The remaining negative cases were localized more anterior or posterior to sections A or section F, respectively (4 cases). It may be mentioned that the labeled fiber tracts originating in these "negative" cases never joined, but occasionally surrounded the MS-VDB complex if the PHA-L deposits were centered in close proximity of AChE-rich cell groups. Contrary to this, PHA-L injection sites within the complex labeled fiber tracts rather selectively within the system giving off a number of synapses in the trajectory through the MS-VDB towards the hippocampus. The very same injections also resulted in extensive projections to the hippocampus. The overall innervation patterns in ammon's horn and dentate gyrus originating from PHA-L injections in MS and VDB are summarized in Table 1.

General Pattern of Hippocampal Innervation

The density of terminal innervation in the hippocampus was evaluated semiquantitatively (Table 1) in those experiments in which PHA-L injections were located within MS or VDB (see Fig. 1). Since iontophoretic delivery of tracer resulted in a precisely localized labeling of a small and limited

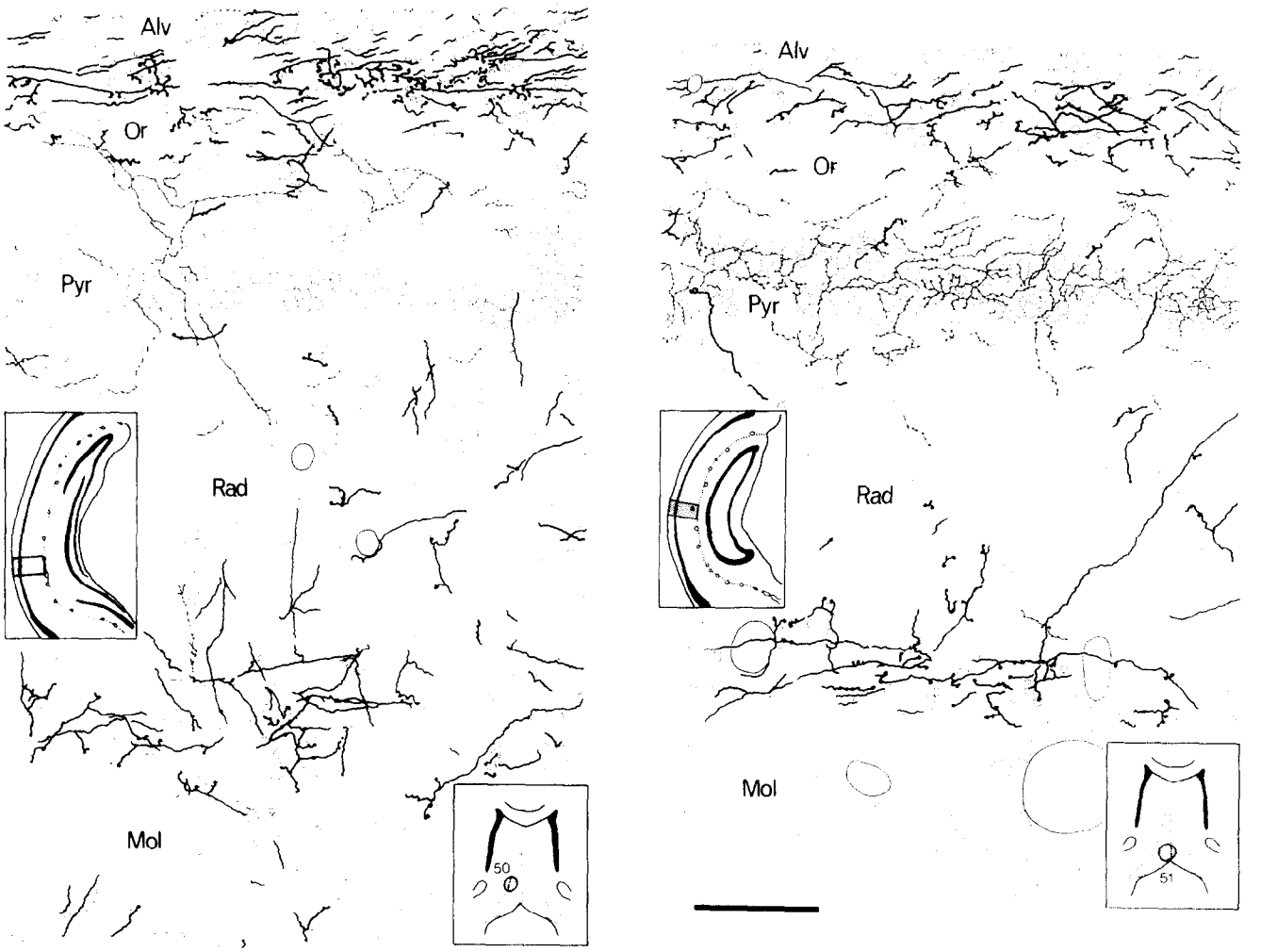


FIG. 4. Comparison of terminal projections in the ventral hippocampal CA1 area from fibers originated from the lateral cell groups of MS (50, left) and from VDB (51, right). The fine fiber network around the pyramidal cells is much more dense in the VDB case. Heavily labeled fibers form bands in the deep part of stratum oriens and of stratum moleculare. Bar=100 μm.

part of the MS-VDB complex, each case showed characteristics of individual neural projections. Furthermore, an attempt was made to draw general conclusions as to the topographic organization of the septo-hippocampal pathway.

Overall pattern of MS projections to the hippocampus. The MS cases were divided into medial-lateral and lateral groups. The majority of MS cases belonged to the medial-lateral group, in which PHA-L injections invaded *both* the medial (primarily non-cholinergic) and lateral (cholinergic) part of the nucleus partly or completely. Correspondingly, the lateral group consisted of those experiments in which the tracer deposit covered the lateral (primarily cholinergic) part only.

The MS experiments are listed from left to right according to the medial-to-lateral positioning of PHA-L injections. Listing of VDB cases from left to right follows mainly the anterior-to-posterior position of the tracer deposit. Thus, VDB divisions are formed as rostral (anterior to MS), intermediate (approx. parallel to the MS), and caudal (angular wing of VDB).

Although semiquantitative evaluation of innervation density was carried out alongside the septo-temporal axis, the projections are differentiated themselves dorso-ventrally, although in several cases a strict dorsal-ventral distinction was rather artificial. Differences in innervation were usually subject to gradual dorso-medial changes in intensity. It is also obvious that some injections stained terminal fibers almost exclusively in either the dorsal (Exps. 14, 44, 65, 63), or the ventral (Exps. 50, 48, 7, 71, 72) hippocampus. In the majority of the experiments, however, fibers and nerve endings could be detected in both hippocampal regions and maximum innervation density was often seen in the posterior (approx. the middle) part of the hippocampus. In agreement with studies using retrograde labeling from the hippocampus, the lateral part of MS exclusively projected to the ventral half of the hippocampus (see Exps. 50, 48, 7). If the injections infiltrated the medial MS, projections extended also into the more dorsal (rostral) part of the hippocampus, which indicates that the medial MS projects to the dorsal hippocampus. There were no significant differences in innervation patterns between the more dorsally (Exps. 29, 28, 27) or ventrally (Exps. 18, 8, 26) located injection sites within the medial-lateral group.

Overall pattern of VDB projections to the hippocampus.

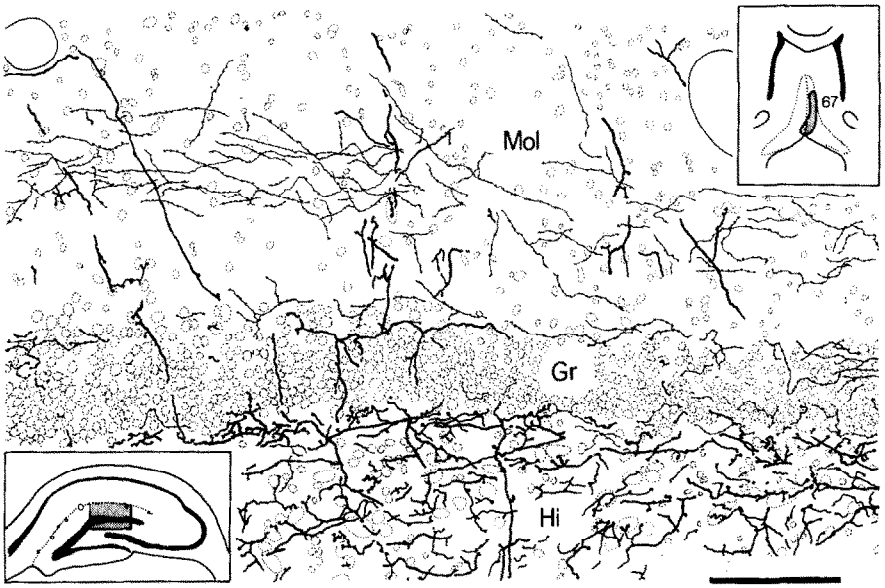


FIG. 5. Characteristics of septal afferent innervation to the dentate gyrus as exemplified in the inner blade at the dorsal hippocampal level. In case 67 a multiple PHA-L injection was performed which invaded both MS and VDB. Apart from the rich supply of the hilar region, the sub- and also supragranular bands are visible. In the middle third of the molecular layer a band of fine fibers is present with numerous en passant synapses. Scale bar=100 μm.

The rostral VDB cases 14 and 44 were located more medial and ventral than 45 and 55 (see Fig. 1A and B). In the former, anterograde labeling occurred only in the dorsal hippocampus, whereas in the latter, the ventral half of the hippocampus was also supplied with labeled efferents. Case 70 received a large injection, involving both medial and more lateral located AChE-rich VDB neurons. The terminal arborization pattern in that case could be detected over the entire septo-temporal axis of the hippocampus.

The VDB cases in the intermediate group, similar to the medial-lateral MS cases, yielded widespread hippocampus projections (Exps. 51 and 31). Injections which invaded the medial column in the posterior VDB (see Fig. 1D and E, cases 71 and 72) resulted in labeling of fibers in the ventral aspect of the hippocampus. Two cases were centered in the caudal tail of the VDB (Exps. 65 and 63, Fig. 1D–F) which both were followed by strong labeling of a practically exclusive dorsal hippocampal projection.

Comparison of innervation of the regio superior vs. regio inferior. In the second step of evaluation of terminal projection fields, the innervation of the two main subdivisions of the hippocampus proper, i.e., regio superior and inferior were compared and related to the supply of efferents to the dentate gyrus. The comparisons were based on the innervation density of different layers, which also allows certain topographic considerations with respect to the source (MS vs. VDB) of innervation (Table 1).

The overall differential pattern of innervation of the various layers was more widespread in the regio inferior (CA3) than in the regio superior (CA1). First of all, the innervation of stratum oriens and the pyramidal cell layer merit attention. Due to the stream of fimbrial fibers passing through CA2 and CA3 regions, the presence of efferents in the oriens and the pyramidal cells layer of the regio inferior is very dense and hard to distinguish from the terminal innervation in other layers of this region. In contrast to this, the innervation of the CA1 pyramidal cell layer seems to be more localized, which is most conspicuous in the dorsal hippocampus (Fig. 3). A second striking difference in the innervation of pyramidal cells in the CA1 of the dorsal hippocampus was found between the MS and VDB experiments. While the CA1 innervation originating from the MS is of minor importance (Fig. 3, left panel), there is a substantial input from VDB (right panel), especially in cases with exclusive dorsal projections (Exps. 44, 63, 65).

A third prominent feature of the innervation of the regio superior is a strongly labeled band of terminal fibers in the deepest part of the stratum moleculare, which probably also covers the superficial part of the stratum radiatum. This fiber tract corresponds strikingly well with an AChE-positive layer described in the stratum lacunosum [11,44]. It seems to be a rather consistent feature that the pattern of efferents to molecular and pyramidal layers in CA1 of the dorsal hippocampus appear simultaneously in most of the experiments.

The innervation pattern in the dentate gyrus (DG) also appears to be subject to topographic organization principles. While supply of input to the ventral DG is rather similar in most of the VDB and the lateral MS cases, this similarity in the dorsal DG only applies to the hilar region. It was obvious that the molecular and granule cell layers towards the septal pole of the DG receive much more of their innervation from the VDB (Table 1).

Detailed innervation pattern in CA1 (regio superior) (Figs. 3, 4, 7). The regio superior was selected to demonstrate the innervation pattern in detail at a cellular level. Individual experiments with innervation in the dorsal and ventral aspects of the hippocampus are shown in Figs. 3 and 4. All layers received input from MS-VDB but the distribution of fibers and especially fibers provided with nerve endings followed a clear laminar arrangement and seemed to aim at different morphological targets.

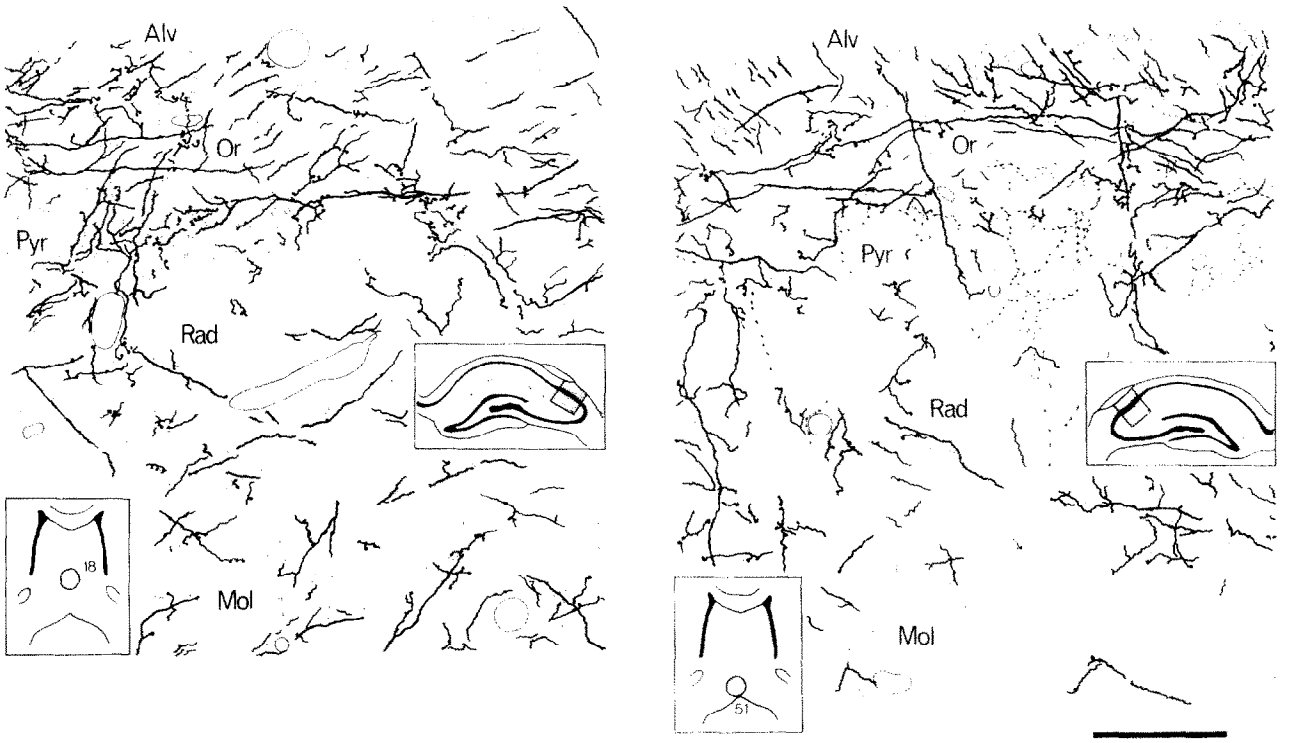


FIG. 6. Terminal innervation patterns in the regio inferior (CA2 and CA3) of the dorsal ammon's horn from the MS (Exp. 18, panel left) and from the VDB (Exp. 51, right panel). Note the abundant fiber network in the stratum oriens and the considerable supply of efferents to the pyramidal cell layer by coarse fibers. The so-called type II fine fibers could only be identified in the VDB case. Calibration bar=100 μm.

One of the most striking aspects of CA1 innervation is that more than one single fiber system arrives from the MS-VDB complex. A very thin and delicate, possibly unmyelinated fiber network could be clearly distinguished from a second much thicker axonal system, which could be easily recognized even with low magnifications. The main target of the fine fibers provided with numerous *en passant* presynaptic boutons, which was tentatively called the type II fiber, appear to be the somata of the pyramidal cells (see Figs. 3 and 4, right sides). Scattered type II fibers, however less abundant could also be observed in other layers as stratum oriens, radiatum and moleculare. Similar to the coarse type I fibers, they are concentrated in the deeper part of the molecular layer (Fig. 3, right panel). In summary, it can be concluded that the thin type II fibers are present as relatively dense networks both in dorsal and ventral CA1 where labeled fiber layers can be seen in the subpyramidal layer and in the transition zone between stratum moleculare and stratum radiatum. A second important conclusion was the observation that after punctate PHA-L injections in the MS-VDB region, anterograde fine fiber labeling in a certain segment of CA1 almost never occurred simultaneously in the subpyramidal and molecular layers. This probably means that individual MS-VDB neurons have spatially dissociated targets with respect to innervation of pyramidal cell bodies or their dendritic fields. Or in other words patches of pyramidal cell bodies receive input from different cell groups in the MS-VDB region than do their dendrites. This differential spatial organization of the septo-hippocampal projection is also one of the reasons that in small injections of tracer, as illustrated in Figs. 3, 4 and 6, anterograde labeling of fine fibers is predominantly present in the pyramidal layers only.

The remaining type I fibers appeared to be much thicker, occasionally showed varicosities, and carried large boutons in most cases on a short stalk. They were mainly concentrated in the deeper parts of stratum moleculare and stratum oriens and formed more or less separate layers running parallel with the laminar orientation. Contrary to this, the thick type I fibers in strata radiatum and pyramidale were mainly oriented orthogonally to the lamina. One and possibly the main target of the coarse fiber system in all layers were interneurons (Fig. 7). This might apply to the pyramidal layer as well, since it also contains interpolated interneurons [39]. In this respect it should be mentioned that the pyramidal layer of the regio inferior is heavily infiltrated with coarse fibers, but any generalization to all CA sectors would be premature at the moment.

The distribution of thick fibers in CA1 shows that their origin within the MS-VDB complex is more widespread than that of thin fibers (Figs. 3 and 4). The source of the type II fibers in the dorsal CA1 projection is exclusively found in the VDB (compare the two sides of Fig. 3). The more posterior and temporal CA1 receive additional innervation from the lateral (cholinergic) part of the MS, which is, however, much less abundant than that from the VDB (Fig. 4).

Detailed pattern of innervation in the dentate gyrus (Fig. 5). The detailed innervation pattern in the dentate gyrus will only be briefly surveyed here. An example of innervation of the DG is shown in Fig. 5. In this experiment (67, not listed in Fig. 1 because it received multiple PHA-L injections), MS and VDB injections were combined to gain a more integral innervation pattern within a single experiment. The pathways of thin fibers could be clearly distinguished here also from the thicker type I axons, first of all because of the

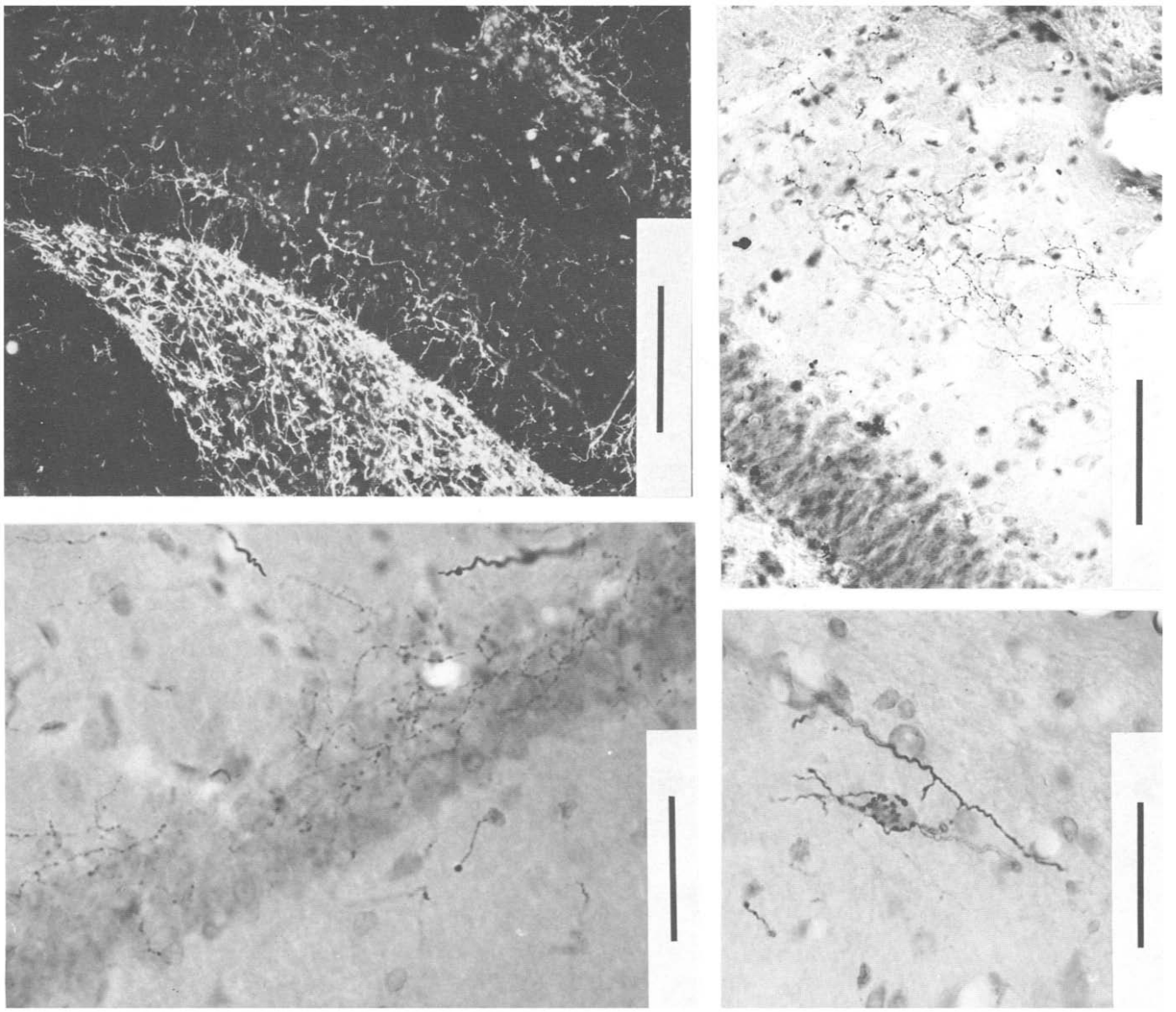


FIG. 7. Top left: darkfield photomicrograph of PHA-L labeled fibers in the dorsal dentate gyrus following a multiple tracer injection in the MS-VDB region (Exp. 67, see also Fig. 5). One can easily discern the heavy labeling in the hilar region and the laminar arrangement of fibers in the granular and molecular layer; Bar=250 μm. Top right: detail of thin PHA-L labeled type II fibers in the middle third of the dentate molecular layer (compare to Fig. 5). Scale bar=100 μm. Bottom left: labeled type II fine fiber network in the subpyramidal layer of CA1 after a tracer injection in the MS as indicated in Figs. 3 and 4. Bar=50 μm. Bottom right: axo-somatic projections involving labeled coarse type I fibers in the stratum oriens of CA1. Calibration bar=50 μm.

differences in topographic arrangement. It was obvious that the middle third of the molecular layer was the major recipient in the dentate gyrus inner blade, which was largely due to innervation with fine-calibre type II fibers (Fig. 7). Regarding the possible number of synapses present in this apical dendritic field, the greater majority of tentative presynaptic endings is supplied by the thin fiber network since the remaining coarse fibers only carry limited numbers of boutons, usually identified in association with interneurons of the DG molecular layer. In contrast, the fine fibers do not seem to be related to cell bodies but give the impression of infiltrating the presumably dendritic fields of the stratum moleculare. The coarse fibers traverse the granular layer and apparently contribute to the innervation of the hilus. Some of these axons arrive medially from the direction of the fornix superior through the hippocampal fissure (for example, the two heavily labeled fibers in the left upper part of Fig. 4). In several experiments it could be observed that the fine fibers formed networks around the granule cell layer, i.e., sub- and supragranularly. As is the case with innervation of the pyramidal cells in CA1, the granule cells are also surrounded by these fine calibre axons infiltrating the cell layer itself. In the section drawn Fig. 5, mainly the supragranular type of innervation patterns is prominent, but some sub- and intragranular arborizations can be discerned as well.

Innervation pattern in the regio inferior (CA3) (Fig. 6). Since fimbrial fibers coming via the alveus infiltrate the hippocampus by traversing the regio inferior as fibers of passage, the laminar arrangement of afferentation to this area is

hard to discern. Although both type I and II fibers can be observed they appear to be so intensely intermingled that a distinct pattern of lamination is difficult to distinguish. In any case, the most abundantly innervated layer is clearly seen in the stratum oriens. From there, fibers can be observed to traverse the pyramidal layer and are continuous with a loosely arranged mainly coarse fiber pattern in stratum radiatum and moleculare-lacunosum. This stream of passing fibers frequently can be followed towards the hilus of the dentate gyrus and to the molecular layer of CA1. Labeling of fine type II fibers, in the dorsal CA3 only seen in VDB injection cases, appears to be limited to the pyramidal cell layer and its adjacent neuropil (Fig. 6, right panel).

DISCUSSION

The aim of the present study was to describe the innervation patterns of hippocampal afferentation from the MS and VDB with special reference to the CA1 region. Despite previous dispute with regard to septal input to CA1 and also to the the dentate gyrus, the unequivocal existence of a differentiated projection from MS/VDB to the CA1 region and other hippocampal regions could be demonstrated by the recently developed sensitive PHA-L tracing method. As will be argued below, it was possible to show that all layers known to display AChE [11, 21, 44] or ChAT [12,15] activity show a remarkable coincidence with labeling of efferents from MS-VDB cells. Moreover, the patterns of lamellar organization of PHA-L positive fibers and terminals are well comparable to the topographic distribution of the cholinergic marker enzymes. In this respect a thin fiber system, which we called the type II fiber, should receive particular attention, since it projects heavily to the pyramidal and granule cell layers of the hippocampus and probably also to the dendritic fields of these neurons. In the CA1 region these type II fibers form the most extensive innervation networks most expressively in the subpyramidal zone, and less densely in supra- and infrapyramidal layers and the inner zone of the stratum moleculare-lacunsum. In the dentate gyrus, the most heavily supplied sublayer is located supragranularly, but there is sub- and infragranular type II innervation as well. The molecular layer of the dentate gyrus also has type II fiber supply in all three sublayers, i.e., outer-, middle-, and inner sublayer, but the fine fiber network dominates in the middle third.

It may well be assumed that the type II fibers are cholinergic in nature. Indirect evidence for this assumption comes from the following observations: (1) type II fibers become labeled after PHA-L injections in areas where the density of AChE-rich neurons (but also ChAT-positive neurons) is highest, i.e., the VDB and the lateral half of the MS [1, 26, 38]; and (2) the innervation patterns show striking coincidence with the distribution of ChAT immunostaining around the pyramidal and granular layers [15]. Since these thin, probably unmyelinated fibers bear numerous boutons in their projection fields, one can conceive their considerable functional significance in the hippocampal afferentation.

The remaining PHA-L labeled fibers (type I) that project to the hippocampus appear to be much thicker, bearing much fewer but larger boutons and innervate essentially all sectors of the hippocampus outside of the pyramidal and dentate granule cell layers. The most dense innervation occurred in the hilus, in all layers of the regio inferior, and in the oriens and molecular layers of CA1. The neuronal source of type I fibers is widely distributed within the MS-VDB complex, which does not permit any conclusion with respect to their transmitter nature. It is likely, however, that both cholinergic and non-cholinergic fibers are included. The possibility that the type II fibers are axon terminal side branches of the type I fibers is unlikely. There is a consistent differentiation in type I and II distribution patterns and in none of the layers a structural link can be observed between thick and thin fibers.

Previous studies on MS/VDB projections employing anterograde axon degeneration and tritiated amino-acid transport techniques agreed on the existence of a dense innervation in the CA3 and CA4 regions, the hilus of the dentate gyrus, and stratum oriens of the CA1 region [21, 25, 27, 29, 36, 37]. Probably as a result of the lesser sensitivity of these methods though, other fields of hippocampal terminal innervation have either been neglected or have been described as projections of minor importance. This is in particular the case for the middle third layer of the stratum moleculare of the dentate gyrus, the stratum moleculare, lacunosum and radiatum of CA1 [21,27], and the supragranular zone [27,29]. It is likely that with the use of degeneration and autoradiographic techniques, only the coarse type I fiber system could be detected more or less successfully, while the type II fibers have remained unobserved. In the present study a detailed and complete description is given of afferentation patterns that all appear to be coexistent with the lamellar distribution of histochemically demonstrated cholinergic marker enzymes. In other words it has been shown here that all AChE and ChAT positive layers and sublayers [11, 15, 44], without exception, receive afferent fibers from the MS-VDB complex.

The regio superior has a unique feature as compared to the regio inferior: its densely packed pyramidal cell bodies, which are traversed by only a few large myelinated fibers. The two main septo-hippocampal pathways, the fornical and fimbrial fiber tracts [18], reach the ammon's horn by invading the CA1 area from both medial and lateral sides. From the lateral aspect, axons pass through the loosely arranged pyramidal cell layer of CA2 and CA3 and follow their way through the dendritic fields of the regio inferior to the hilus and molecular layer of the dentate gyrus, but also toward the CA1 molecular layer. This anatomical organization of fiber trajectory may be one of the main reasons why the laminar distribution of fibers is more clearly recognized in CA1 than in other sectors of the hippocampus proper. This applies to the deep part of molecular-lacunar layer where the fibers run perpendicular to the apical dendrites of pyramidal cells and form a pathway which corresponds well to the AChE- [44] and ChAT- [15] positive layer described by other authors. Since the CA1 pyramidal layer is relatively free of large crossing fibers, its input formed by the fine type II fiber system could also be more easily discerned. These type II fibers are also present in the regio inferior, but are more difficult to distinguish, because of the intermingling in this area between type II and type I fibers, the latter infiltrating and passing through the pyramidal layer.

The innervation patterns of the MS-VDB projections and their laminar organization in the fascia dentate also matches very well with the distribution of AChE and ChAT activity. The supply of efferents to the molecular layer is widespread but the middle third receives the strongest input. Furthermore, there is a second major band of innervation in the supragranular zone primarily by type II fibers, which are continuous with a sparse innervation of the granular layer. In the subgranular zone, the type II fibers mix with the thick

type I terminal branching, which becomes extremely dense and randomly organized in the hilar region. This hilus receives afferentation through both the dorsal fornix and the fimbria. The fimbrial input divides in two fibers tracts, the more extensive pathway running through the dendritic fields of CA3 and CA4 pyramidal cells, and the minor one reaching the hilus via an infrapyramidal route. The fornical fibers cross the hippocampal fissure and all layers of fascia dentata. On their way to the hilus, the latter fornical fibers form synapses in various sublayers, like the middle third of the molecular layer of fascia dentata (Fig. 5).

In agreement with previous observations [18,27], the MS-VDB input pathways follow through the two main fimbrial and fornical channels, providing efferents directly to the entire hippocampus or indirectly by traveling over some distance in the alveus. The topographically ordered innervation along the septo-hippocampal axis depends largely if not entirely on the balance between the fornical vs. fimbrial routes. This innervation pattern is, of course, determined originally by the location of innervation neurons within the MS-VDB complex as revealed by studies using retrograde labeling techniques [1, 18, 22, 26, 38, 50]. The present results confirm the existence of a topographic organization of medial vs. lateral MS and anterior vs. posterior VDB cells projecting to dorsal vs. ventral parts of the hippocampus, respectively. Furthermore, we stress that not only the lateral MS but also the posterior aspects of the MS and the posteriorly located neurons of intermediate parts of the VDB (which actually form the ventral continuation of the posterior MS) send fibers to the more temporal aspect of the hippocampus. The origins of fibers supplying the dorsal hippocampus are in the medial MS, the medioventral aspects of VDB, and the caudal part of VDB.

The considerable topographic difference in the origin of the septo-hippocampal afferentation with respect to the innervation along the septo-temporal axis argues against the unitary theories in the explanation and understanding of the hippocampal function. If ventral and dorsal hippocampus are contrasted, a distinction can also be shown in behavioral [30], endocrine [6], and neurochemical [3] parameters.

Functional Considerations

The function of the hippocampus has long been coupled to cognitive, learning and memory functions and more specifically to spatial orientation and learning [16, 32–34, 48]. Spatial learning and memory are thought to require a much more localized neural input than what was offered by previous anatomical descriptions of septo-hippocampal afferentation [21,33]. The results of the present study suggest that such a localized spatial organization of projection patterns is present indeed. This may primarily involve the very thin unmyelinated fibers, because it is mainly this type II fiber system which appears to be topographically organized both with respect to its origin in MS-VDB and its final termination pattern. The innervation of pyramidal and granular cell layers is arranged in rather narrow segments. Moreover, from our tracing experiments it may be concluded that individual MS/VDB neurons never supply efferents simultaneously to cell bodies and their dendritic fields in the same segment of cornu ammonis or dentate gyrus. This means that within a certain hippocampal segment dendrites and cell bodies receive thin fiber input from topographically different cell groups of the MS/VDB complex. The transmitter nature of this fine fiber afferentation, as it was indirectly argued, most probably is cholinergic. The hippocampal cholinergic mechanisms are part of the basal forebrain cholinergic systems, which play a key role in learning and memory processes [8,41], a conclusion strongly substantiated by the results of extensive research on the causal factors related to dementia of the Alzheimer type [41,47].

The other important aspect of hippocampal function is the display of synchronized rhythmic slow electric activity (theta), which is triggered by the MS-VDB neurons [4, 7, 33]. The theta rhythm also has a cholinergic component apart from the movement-related non-cholinergic component [7,51]. The existence of more than a single afferent fiber system may offer further support in the understanding of the multiplicity of rhythmic slow activity: e.g., cholinergic vs. non-cholinergic, and CA1 and dentate gyrus generated electrical synchronization [5, 14, 31, 49, 51, 52]. While the molecular layer is claimed to be the locus of theta generation in the fascia dentata [5,31], there is electrophysiological evidence for a direct excitation of granule cells just above the soma [23], i.e., in the supragranular zone, which receives a considerable cholinergic innervation and fiber supply. Of particular interest in this respect is the double dipole model of theta generation [14], according to which the generators are close to the pyramidal and granule cell layers and should be supplied simultaneously with a monosynaptic pathway from the septum. This hypothesis is supported by our observation that the type II fibers provide parallel innervation both to the pyramidal and granule cells.

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